Immunology 2004 **112** 567–574 doi:10.1111/j.1365-2567.2004.01916.x

Ubiquitin-fusion degradation pathway plays an indispensable role in naked DNA vaccination with a chimeric gene encoding a syngeneic cytotoxic T lymphocyte epitope of melanocyte and green fluorescent protein

MANXIN ZHANG,* KAZUNARI ISHII,* HAJIME HISAEDA,* SHIGEO MURATA,‡ TOMOKI CHIBA,‡ KEIJI TANAKA,‡ YANG LI,* CHIKAGE OBATA,† MASUTAKA FURUE† & KUNISUKE HIMENO* *Departments of *Microbiology and Immunology, †Dermatology, Graduate School of Medical Sciences, Kyushu University, Fukuoka, Japan and ‡Department of Molecular Oncology, The Tokyo Metropolitan Institute of Medical Science, Tokyo, Japan*

SUMMARY

Antitumour immunity against murine melanoma B16 was achieved by genetic immunization with a naked chimeric DNA encoding a fusion protein linking green fluorescent protein (GFP) to the N-terminus of a major $CD8^+$ cytotoxic T lymphocyte (CTL) epitope of tyrosinase-related protein 2 (TRP-$2_{181–188}$) of murine melanoma, designated as pGFP-TRP-2. Tumour growth was profoundly suppressed in C57BL/6 mice immunized with pGFP-TRP-2, while mice vaccinated with pTRP-2 showed rapid tumour growth and died within 40 days after tumour challenge. Splenocytes of mice immunized with pGFP-TRP-2 showed high CTL activity specific for TRP-$2_{181–188}$. GFP-TRP-2 expressed in COS-7 cells was rapidly degradated *in vitro* and the degradation was almost completely prevented by adding a proteasome inhibitor, MG-132, in the culture. Furthermore, the antimelanoma immunity induced by genetic immunization with pGFP-TRP-2 was completely cancelled in mice deficient in proteasome activator PA28α/β. Taken together, GFP-TRP-2 processed by cytosolic proteasome played a central role in breaking peripheral tolerance to a melanoma/melanocyte antigen, TRP-$2_{181–188}$, by activating $CD8^+$ CTL specific for TRP-$2_{181–188}$. TRP-$2_{181–188}$ fused to GFP may be readily cut off from GFP by the ubiquitin-fusion degradation (UFD) pathway and efficiently presented to major histocompatibility complex class I molecules, resulting in effective induction of $CD8^+$ T cells specific for the CTL epitope. Furthermore, $CD4^+$ T cells specific for GFP were shown to play a crucial role in the antimelanoma immunity, probably potentiating activity of TRP-2-specific CTL and/or the 'ubiquitin-proteasome pathway'. It is noteworthy to document that genetic immunization with pGFP plus pTRP-$2_{181–188}$ failed to exert the antitumour immunity.

Keywords Melanoma, UFD pathway, TRP-2, DNA vaccine, GFP

INTRODUCTION

Antigen presentation to $CD8^+$ T cells is mediated by major histocompatibility complex (MHC) class I molecules expressed on antigen-presenting cells (APC)/dendritic cells (DC). Primarily, $CD8^+$ T cells recognize MHC class I-associated peptides derived from endogenous antigens, such as oncogene-products or viral antigens, located in the cytosol. Prior to antigen presentation by MHC class I molecules, antigens must be ubiquitinated and then be processed into antigenic peptides by the proteasome. This antigen processing system is termed as 'ubiquitin-proteasome pathway'.[1–3] Vaccination with naked DNA encoding endogenous antigens is an efficient means for induction of antigen-specific immunity, particularly activation of $CD8^+$ T cells, as those intracellularly expressed gene product, antigenic protein, must be exclusively processed through the ubiquitin-proteasome

Received 9 October 2003; revised 26 March 2004; accepted 5 May 2004.

Correspondence: Dr K. Himeno, Department of Microbiology and Immunology, Graduate School of Medical Sciences, Kyushu University, 3-1-1 Maidashi, Higashi-ku Fukuoka 812-8582, Japan. E-mail: himeno@parasite.med.kyushu-u.ac.jp

pathway and the created MHC class I-binding epitopes activate specific $CD8^+$ T cells.

A large number of melanoma antigens able to activate immune responses have been identified.[4–6] Among them, melanocyte lineage differentiation antigens (MDAs) are the most prominent in induction of immune responses. The tyrosinase-related protein 2 (TRP-2) is one of MDAs expressed by normal and malignant melanocytes both in humans and mice.[7,8] Several epitopes recognized by human $CD8^+$ T cells have been identified on the TRP-2 protein.[9] Bloom *et al.* identified a major cytotoxic T lymphocyte (CTL) epitope, TRP-$2_{181–188}$, recognized within the H-$2K^b$ MHC class I molecules, and they reported that passive transfer of TRP-$2_{181–188}$-specific CTLs into C57BL/6 (B6) mice is therapeutic against established B16 lung metastasis.[8] Naive B6 mice have significant numbers of CTL precursors with reactivity to TRP-$2_{181–188}$. However, the immune response to TRP-$2_{181–188}$ may be limited by the tolerance mechanisms that only allow low-affinity lymphocytes to survive after T-cell selection in the thymus.[10] Overcoming these tolerizing limitations may be critical to the development of clinical immunotherapeutic approaches for patients with melanoma. Self-tolerance is broken down in certain conditions. One case is that self-antigens are effectively presented to MHC class I molecules on professional APC/DC, then those tolerant $CD8^+$ T cells are activated.[11] Another case is that $CD4^+$ T cells recognizing MHC class II-associated peptides of target antigens support to break the tolerance of $CD8^+$ T cells specific for autologous tumour antigens via releasing some cytokines such as interleukin-12 (IL-12), probably through activation of DC.[12]

Previous experiments demonstrated that genetic immunization of B6 mice with cDNA encoding autologous murine TRP-2 as a model antigen was unable to induce significant protective immunity against B16 melanoma cells naturally expressing TRP-2.[13] We supposed that failure in breaking peripheral tolerance of $CD8^+$ T cells by ordinary DNA vaccinations is explained in two ways. The first cause may be that tag-molecules which lead antigenic peptide to the ubiquitin-proteasome pathway are not contained or insufficient in some antigenic proteins. The second cause should be that MHC class II-binding epitopes are not contained in the TRP-2, especially TRP-$2_{181–188}$. Green fluorescent protein (GFP) is known to be readily targeted by ubiquitin fusion degradation (UFD) pathway, a virtual route of the ubiquitin-proteasome pathway.[14]

In this study, we investigated the effect of DNA vaccination with a gene encoding TRP-$2_{181–188}$ fused to GFP (pGFP-TRP-2) on murine melanoma tumour growth *in vivo* using B16 cells, a spontaneous and poorly immunogenic murine melanoma cell line. Strikingly, immunization with pGFP-TRP-2 induced potent protective immunity against the tumour. It is noteworthy that immunization neither with pTRP-$2_{181–188}$ alone nor with pGFP plus pTRP-$2_{181–188}$ induced the antitumour immunity. A key role of the ubiquitin-proteasome pathway in the present study was confirmed *in vitro* using a proteasome inhibitor, MG-132 and *in vivo* using proteasome activator PA28α/β-deficient mice.

MATERIALS AND METHODS

Animals and tumours

We performed mouse studies in accordance with the institutional guidelines of Kyushu University. Female C57BL/6 (B6) mice between 7 and 8 weeks of age were purchased from Japan SLC, Inc. (Hamamatsu, Japan). Proteasome activator PA28-knockout (PA28$\alpha/\beta^{-/-}$) mice of B6 background were also used.[15] B16F1 and B16F10, murine melanoma cell lines, were maintained *in vitro* in RPMI-1640 supplemented with 10% fetal bovine serum, 100 IU/ml penicillin, 100 μg/streptomycin, 20 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulphonic acid, 50 mM $NaHCO_3$ and 2 mM L-glutamine.

Plasmids

pGFP plasmid, purchased from Clontech (Palo Alto, CA), was used to construct GFP-TRP-2 plasmid: GFP-TRP-$2_{181–188}$ DNA was amplified by PCR using sense 5′-CAG AGAATTCAGACGCCACCATGGTG-3′ and antisense 5′-CCATGGATCCTATTAGAGCCACACAAAAAAGT CATACACCTTGTACAGCTCGTC-3′ primers and using pGFP plasmid DNA as a template. The PCR product of GFP-TRP-$2_{181–188}$ DNA was inserted into *Eco*RI and *Bam*HI sites of a pcDNA 3.1 (–) vector (Invitrogen, San Diego, CA). A pTRP-2 plasmid encoding TRP-$2_{181–188}$ was constructed as follows: DNA was amplified by polymerase chain reaction (PCR) using sense 5′-CAGAGAATTCA AAGGCATGGTGTATGACTTTTTTGTGTGGCTCTA ATAG-3′ and antisense 5′-ACGTCTTAAGAGATATCCA GCACAGT-3′ as primers and using pGFP-TRP-2 plasmid DNA as a template. After treatment with *Dpn*I, the PCR product was ligated and used for transformation of *Escherichia coli*.

In vivo *gene transfer and implantation of melanoma cells*

We used a Helios Gene Gun (Bio-Rad, New York, NY) as previously described.[16,17] Briefly, plasmid DNA was precipitated onto 1·6 μg gold particles and coated on the inner surface of tubing by a tube loader. The final tubing segment enabled delivery of 0·125 mg gold particles and 2 μg plasmid DNA per transfection. pTRP-2, pGFP-TRP-2 or pGFP were transferred to three different portions of shaved abdominal skin three times at 1-week intervals. A total of 18 μg plasmid was administered to each mouse. One week after the last vaccination, 2×10^5 melanoma cells in 0·1 ml of phosphate-buffered saline (PBS) were injected subcutaneously (s.c.) into each B6 mouse. Tumours were measured twice a week using a caliper, and tumour volume was calculated as $\pi/6 \times [(a \times b)^{1/2}]^3$, where a and b are two perpendicular major diameters.

In vitro *transfection and Western blotting*

Five hundred thousand COS-7 cells in a 2·5-cm dish (Nunc, Roskilde, Denmark) were transfected with 2 μg of each plasmid DNA by using Lipofectamine. Twenty-four hr after transfection, cell lysates were prepared by adding 200 μl lysate buffer (50 mM Tris-HCl/1% Nonidet P-40/1% sodium dodecyl sulphate (SDS)/1 μM

leupeptin/100 μM phenylmethylsulphonylfluoride/1 μM pepstatin A/100 μM ethylenediamine tetraacetic acid), and 10 μg protein was used for Western blotting with anti-HA (mouse monoclonal antibody clone 12CA5, Roche, Mannheim, Germany) diluted at 1 : 2000 as the first antibody. Peroxidase-conjugated anti-mouse immunoglobulin G (IgG) (H + L) (Zymed, San Francisco, CA) was the second antibody. Binding antibodies were detected by using enhanced chemiluminescence (ECL) reagents (Amersham, Amersham, UK). Anti-GFP monoclonal antibody JL8 (Clontech) diluted at 1 : 1000 was used as the first antibody for detecting GFP and GFP-TRP-2 expression. Proteasome inhibitor MG-132 (Bostonchem, Boston, MA) was added at 10 μg/ml 24 hr after transfection, and cell lysates were prepared 24 hr after addition of MG-132.

Cytotoxicity assay

The H-2K^b-binding peptide VYDFFVWL (TRP-2$_{181-188}$) was synthesized by an Fmoc/PyBOP strategy and purified by high-pressure liquid chromatography (SAWADY Technology, Tokyo, Japan). Peptides were dissolved at 10 μg/ml in PBS containing 10% dimethylsulphoxide and stored at −20°. Mice were killed at the time of tumour challenge, and their spleen cells (5×10^6) were cocultured with 10^5 mitomycin C-treated B16F1 cells in 24-well culture plates in complete RPMI-1640 media. After culturing for 5 days *in vitro*, graded numbers of viable effector cells and [^{3}H]thymidine-labeled EL-4 cells (10^4), pulsed for 2 hr with TRP-2$_{181-188}$ peptide (4 μg/ml), were placed into the round-bottomed wells of 96-well plates. After 4·5 hr of incubation, the cells and their media were harvested onto glass-fibre filters, and then radioactivity level was assessed using a beta scintillation counter and specific killing was calculated.

In vivo *depletion of T-cell subsets*

To determine the relative contributions of T cells to antitumour immunity, antibodies corresponding to each T-cell subset were administered to mice. Anti-CD4 monoclonal antibody (mAb; clone GK1.5) or anti-CD8 mAb (clone 53-6.72) was injected intraperitoneally at 0·5 mg per injection per mouse on days −3, −1, 1 and 3. Tumour challenge was on day 0. Depletion of each T-cell subset was confirmed by flow cytometric analysis, and over 95% of the cells of the appropriate subsets were regularly found to be depleted.

Enzyme-linked immunosorbent assay (ELISA)

A 96-well plate (Nunc) was coated with 100 μl of GFP (Clontech) at 1·0 μg/ml in PBS overnight at 4°. To prevent non-specific binding, 200 μl of blocking buffer (0·1% bovine serum albumin, 0·05% Tween-20 in PBS, pH 7·2) was added to a coated plate, and the plate was incubated at room temperature for 1 hr. Then serum samples (each 100 μl) from pGFP-TRP-2-immunized mice and control mice, diluted 1000–27 000-fold with PBST (0·05% Tween-20 in PBS), were placed in duplicate wells. After 1·5 hr of incubation, the plate was washed four times with PBST and then incubated with 100 μl of the second antibody (anti-mouse IgG-horseradish peroxidase, Pierce, Rockford, IL) diluted 1000-fold with PBST for 1·5 hr. After washing five times, bound antibodies were visualized by the addition of a substrate of horseradish peroxidase (OPD, Sigma, St Louis, MO). Intensity was measured as OD at 450 nm by using an ELISA reader (Labsystem Multiskan, Finland).

RESULTS

Construction of plasmids encoding TRP-2$_{181-188}$ or GFP-TRP-2$_{181-188}$

A GFP-TRP-2$_{181-188}$ expression plasmid was constructed by PCR using pGFP as a template and the primers shown in Materials and Methods (pGFP-TRP-2), and a TRP-2$_{181-188}$ expression plasmid was constructed by PCR using pGFP-TRP-2 as a template and the primers shown in Materials and Methods (pTRP-2). Construction of pTRP-2 and pGFP-TRP2 were confirmed by DNA sequencing. The expression of fusion GFP-TRP-2 was confirmed by Western blotting after transfection of COS-7 cells with these plasmids (Fig. 1). A single band was detected in cells transfected with pGFP or pGFP-TRP-2. GFP alone was strongly expressed more than the fusion GFP-TRP-2.

Antitumour effect of gene gun-mediated vaccination with fusion pGFP-TRP-2

To determine the antitumour effect of gene gun-mediated vaccination, we delivered pTRP-2, pGFP plus pTRP-2 or pGFP-TRP-2 into the abdominal skin of B6 mice before challenge with syngeneic B16F1 melanoma cells. Rapid tumour growth was seen in mice vaccinated with control pGFP, and the mice died usually 30–40 days after the challenge, findings similar to those in a group of the mice that had not been vaccinated. Vaccination with pTRP-2 or pGFP plus pTRP-2 did not influence tumour growth and could not rescue mice. Mice vaccinated with pGFP-TRP-2 showed limited tumour growth, a part of which survived with a trace of tumour growth, although some mice gradually died much later than control mice (Fig. 2). Those mice that had rejected B16F1 cells following pGFP-TRP-2 vaccination readily acquired resistance to a challenge even with much higher tumorigenic B16F10 (data not shown), indicating that strong immunity was induced and that immunological memory had been established. These protected mice showed vitiligo-like depigmentation on their skin, evidence of immunity to melanocytes, while mice vaccinated with the control plasmid did not (data not shown). These results demonstrate that fusion of GFP to CTL epitope broke the tolerance to tumour antigen. Tumour growth was also suppressed in mice vaccinated with a single intramuscular injection of pGFP-TRP-2 (data not shown).

Cellular requirement for induction of antitumour immunity by vaccination with pGFP-TRP-2

It is known that tumour-specific CTLs play a crucial role in rejection of a tumour. We therefore carried out an

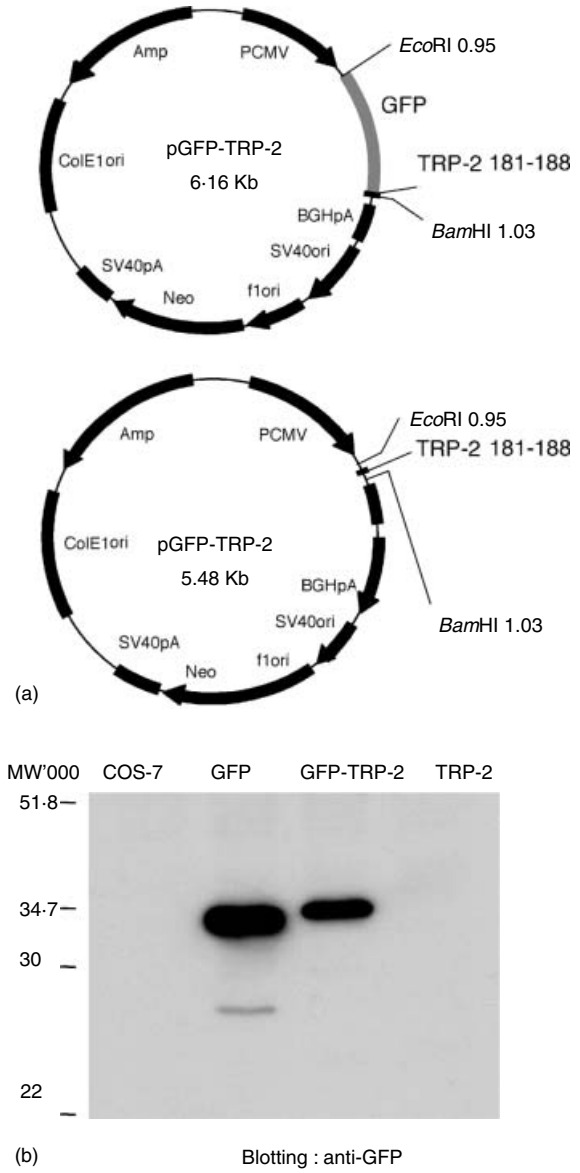

Figure 1. Plasmid construction and expression of GFP-TRP-2 in COS-7 transfectants. (a) Schematic representation of pGFP-TRP-2 (upper panel) and pTRP-2 (lower panel). (b) COS-7 cells were transfected with pGFP or pGFP-TRP-2 using Lipofectamine. Expression of GFP or GFP-TRP-2 was detected by Western blotting with anti-GFP as the first antibody. Sizes of the molecular weight markers are shown on the left. The experiment of (b) was repeated three times with similar results.

experiment to determine whether vaccination could induce CTLs specific for the tumour before the challenge. No activity of CTLs was detected in splenocytes of mice vaccinated with pGFP or even with pTRP-2. In contrast, splenocytes obtained from mice vaccinated with pGFP-TRP-2 showed prominent cytotoxicity to H-2^b-bearing EL4 pulsed with TRP-$2_{181-188}$ peptide (Fig. 3). Thus, vaccination with pGFP-TRP-2 effectively induced specific CTLs, indicating that activation of $CD4^+$ T cells is crucial for induction of CTLs by this vaccination. No evidence of

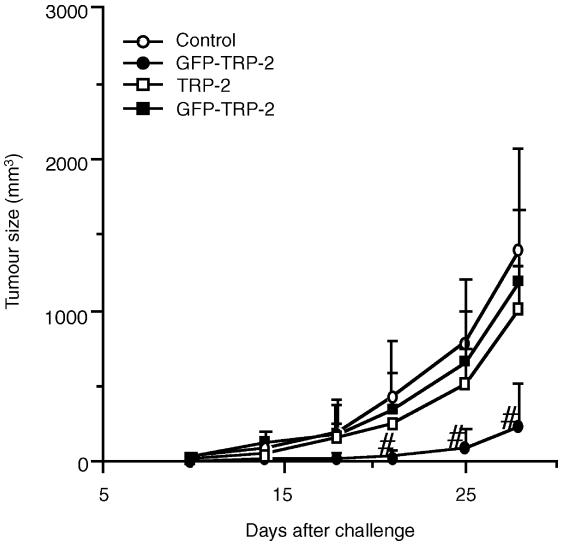

Figure 2. Induction of antitumour immunity against B16F1 in C57BL/6 mice by vaccination with pGFP-TRP-2.C57BL/6 mice were immunized with pGFP (open circles), pTRP-2 (open squares), pGFP-TRP-2 (closed circles) or pGFP plus pTRP-2 by using a gene gun and then challenged s.c. with 2×10^5 of B16F1 melanoma cells. Each value represents the mean ± SD from seven mice. Tumour growth was measured twice a week after challenge. #$P < 0{\cdot}01$ compared with the other two groups, by unpaired Student's *t*-test. This experiment was repeated five times.

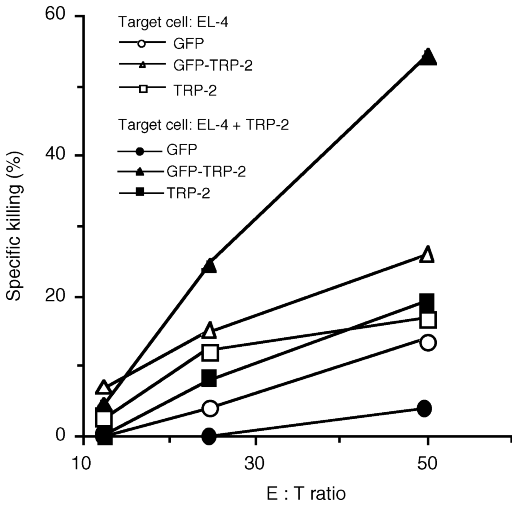

Figure 3. CTL activity induced by vaccination with pGFP-TRP-2. Effector cells were prepared from splenocytes (5×10^6) obtained from immunized C57BL/6 mice after *in vitro* stimulation with mitomycin C-treated B16F1 cells for 5 days. [^{3}H]thymidine-labelled EL-4 cells were pulsed with (closed symbols) or without (open symbols) TRP-$2_{181-188}$ peptide used as target cells. Effector and target cells were incubated for 4·5 hr and then harvested and counted using a beta counter, and specific killing was calculated. This experiment was repeated for three times with similar results.

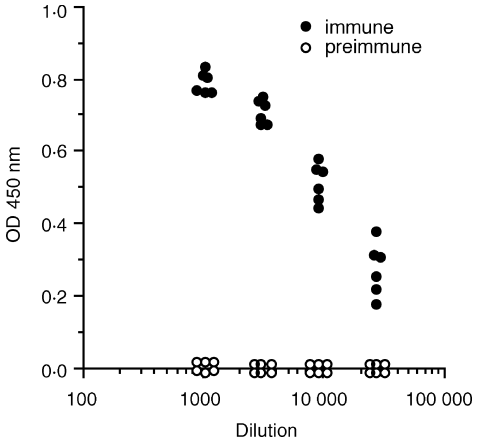

Figure 4. GFP-specific IgG in mice immunized with pGFP-TRP-2.C57BL/6 mice were immunized five times at 1-week intervals by using a gene gun. Serum samples collected from the immunized mice one week after the fifth immunization (closed symbols) were serially diluted, and ELISA was performed on plates coated with recombinant GFP. Sera from preimmune mice (open symbols) were also analyzed. Each symbol represents the result from an individual mouse. This experiment was repeated for three times.

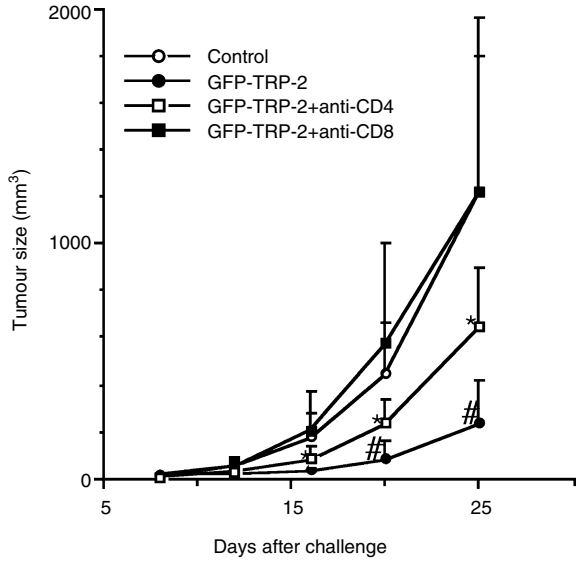

Figure 5. Cellular requirement for antitumour immunity induced by immunization with pGFP-TRP-2. Twenty-one C57BL/6 mice immunized with pGFP-TRP-2 were divided into three groups, one of which was left untreated (closed circles). Two groups of mice were administered with either anti-CD4 (open squares) or anti-CD8 mAb (closed circles) on days −3, −1, 1 and 3. Tumour cells were challenged on day 0. Unvaccinated mice (open circles) were also challenged. Each value represents the mean ± SD from seven mice. $\#P < 0{\cdot}01$ compared with control and anti-CD8 group and $*P < 0{\cdot}05$ compared with untreated immune group, by unpaired Student's *t*-test. This experiments was repeated three times with similar results.

$CD4^+$ T cell responses to TRP-2 was obtained since the plasmid used for vaccination only expressed CTL epitope of TRP-2 (data not shown). However, a foreign protein, GFP, could activate $CD4^+$ T cells as assessed by production of GFP-specific IgG. High levels of GFP-specific IgG antibodies were detected in sera obtained from mice vaccinated with pGFP-TRP-2 (Fig. 4). Because it is known that isotype switching requires help from antigen-specific $CD4^+$ T cells, this finding indirectly confirmed the induction of GFP-specific $CD4^+$ T cells *in vivo*, although we have no direct evidence that GFP recalls responses of $CD4^+$ T cells *in vitro*.

We further investigated the cells responsible for antitumour immunity by carrying out cell-depletion experiments. Mice vaccinated with pGFP-TRP-2 were depleted of a specific cell subset by administration of the corresponding antibodies. After confirmation of successful depletion of the T-cell subset, B16F1 cells were inoculated. As shown in Fig. 5, depletion of $CD8^+$ T cells abrogated the antitumour effect induced by vaccination with pGFP-TRP-2. Tumours in mice depleted of $CD8^+$ T cells developed more rapidly than even those in unvaccinated mice. Depletion of $CD4^+$ T cells also significantly affected the antitumour immunity but to a lesser extent than that of depletion of $CD8^+$ T cells. Thus, both $CD8^+$ and $CD4^+$ T cells were required for induction of antitumour immunity.

Degradation of GFP-TRP-2 by proteasome

If a foreign GFP only functions as an activator of $CD4^+$ T cells, coimmunization with two plasmids expressing either TRP-2 or GFP should be effective. However, our results demonstrated that coimmunization did not induce CTL activity (Fig. 2), strongly suggesting the requirement of a physical linkage between the two proteins and an additional role of GFP in induction of CTLs. We therefore hypothesized that intracellular GFP is rapidly degraded by the ubiquitin-proteasome pathway and the TRP-$2_{181-188}$ fused to GFP is readily cut off from GFP and efficiently presented to MHC class I molecules, resulting in effective induction of $CD8^+$ T specific for the CTL epitope. Therefore, the CTL epitope should effectively associate with MHC class I molecule and then be presented to $CD8^+$ T cell. To prove this, we transfected COS-7 cells with pGFP or pGFP-TRP-2 in the presence of a proteasome inhibitor MG-132 (Fig. 6). Chemical inhibition of proteasome resulted in accumulation of both GFP alone and an epitope fused to GFP, indicating that intracellularly expressed intracellular GFP is susceptible to proteasomal degradation. Thus, antigen presentation of TRP-$2_{181-188}$ to $CD8^+$ T cells became significantly effective by fusion to the foreign protein GFP.

Defective induction of antitumour immunity in PA28$\alpha/\beta^{-/-}$ mice by immunization with pGFP-TRP-2

In order to clarify the role of the proteasome in antitumour immunity induced by pGFP-TRP-2, we employed PA28$\alpha/\beta^{-/-}$ mice, because TRP-$2_{181-188}$ is cut off by PA28-

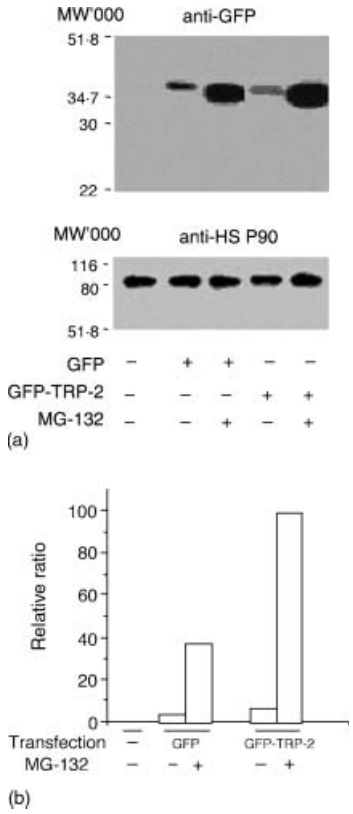

Figure 6. Processing of GFP and GFP-TRP-2 protein by proteasome. COS-7 cells were transfected with pGFP or pGFP-TRP-2 using Lipofectamine. Twenty-four hr after transfection, the proteasome inhibitor MG-132 was added and cells were prepared 24 hr later for detection of GFP and HSP90 by using Western blot. Sizes of molecular weight markers are shown on the left in kilodaltons (a). Relative expression of GFP-TRP-2 to that of HSP90 was calculated densitometrically (b). This experiment was repeated twice.

mediated ubiquitin-proteasome pathway.[15] PA28$\alpha/\beta^{-/-}$ mice immunized with pGFP-TRP-2 were markedly susceptible to B16F1 tumour challenge compared with wild-type B6 mice immunized with the same plasmid (Fig. 7a). On day 14 after s.c. challenge with B16F1, these mice were challenged again with intravenous (i.v.) injection of B16F10 tumour cells, and the number of lung metastases of B16F10 cells was counted three weeks after the i.v. challenge. The number of lung metastases was smaller in pGFP-TRP-2-immunized mice than in control mice ($P = 0{\cdot}02$). The number of lung metastases in PA28$\alpha/\beta^{-/-}$ mice immunized with the same plasmid was slightly less than that in control mice ($P = 0{\cdot}15$) but remarkably greater than that in pGFP-TRP-2-immunized mice ($P = 0{\cdot}06$), although the difference was not significant (Fig. 7b). Coincident with the results of tumour growth, CTL activity against the TRP-$2_{181-188}$ epitope was lower in PA28$\alpha/\beta^{-/-}$ mice immunized with pGFP-TRP-2 than in wild-type mice (Fig. 7c). Similar levels of GFP-specific IgG were detected in PA28$\alpha/\beta^{-/-}$ and wild-type B6 mice after three immunizations with pGFP-TRP-2 (Fig. 7d), indicating that the responses of $CD4^+$ T cells were not affected by PA28α/β deficiency. These results proved that the proteasome activated by PA28 plays an important role in the induction of TRP-$2_{181-188}$-specific CTL and antitumour immunity following DNA vaccination with pGFP-TRP-2.

DISCUSSION

The final goal for tumour immunotherapy is to induce antigen-specific CTL activity. A major obstacle that must be overcame for induction of effective tumour immunity is peripheral self-tolerance to tumour antigens. For this purpose, we constructed a plasmid (pGFP-TRP-2) encoding a fusion protein of a defined CTL epitope, TRP-$2_{181-188}$, to a foreign protein, GFP. GFP is known to be readily targeted by uibiquitin-fusion degradation (UFD) pathway, a virtual route of the ubiquitin-proteasome pathway.[14] Furthermore, GFP has a potential to activate specific $CD4^+$ T cells.[18] In the present study, immunization with pGFP-TRP-2 induced potent antitumour immunity promoted by both $CD4^+$ and $CD8^+$ T cells. Our vaccination with the naked chimeric DNA clearly demonstrated the crucial contributions of the UFD pathway to the antitumour immunity, as evaluated by experiments *in vitro* with proteasome inhibitor MG-132 and *in vivo* with proteasome activator PA28α/β knockout mice.

It is noteworthy that GFP fused to TRP-$2_{181-188}$ expressed within cytosol of APC plays a central role in the induction of TRP-$2_{181-188}$-specific CTLs. Thus, directed linkage between a CTL epitope and GFP should be required to induce TRP-$2_{181-188}$-specific CTLs, since DNA vaccination with pGFP plus pTRP-2 was not effective in inducing the antitumour immunity, as shown in Fig. 2. One interpretation for the necessity of direct linkage between GFP and TRP-2 is that either MHC class II-binding peptide, GFP recognized by $CD4^+$ T cells, or MHC class I-binding peptide, TRP-$2_{181-188}$ recognized by $CD8^+$ T cells, have to be presented by the same APC. Notably, cytosolic foreign proteins which have been monoubiquitinated are processed by lysosomal enzymes.[19] Accordingly, some peptides in those foreign proteins should be presented by MHC class II molecules, which activate specific $CD4^+$ T cells. Another possibility should be that a part of the fusion protein, GFP-TRP-2, generated within APC/DC is secreted into circulation and then pinocytosed/phagocytosed by other APC/DC and then some degraded peptides of GFP should be presented by MHC class II molecules on the APC/DC resulting in an inevitable activation of GFP-specific $CD4^+$ T cells, while TRP-$2_{181-188}$ fused to GFP stimulates its specific $CD8^+$ T cells. As shown in Fig. 1, GFP is susceptible to degradation by proteasome, the initial step of antigen presentation to $CD8^+$ T cells. Endogenous antigens such as tumour antigens is processed by proteasome after ubquitination.[2] Ubiquitin binds target proteins via lysine.[3] GFP contains 19 lysine residues,[20] and is easily degraded by the UFD pathway,[14] which may be very helpful for ubiquitination and antigen processing of the fusion protein GFP-TRP-$2_{181-188}$. Thus, the ubiquitin-proteasome

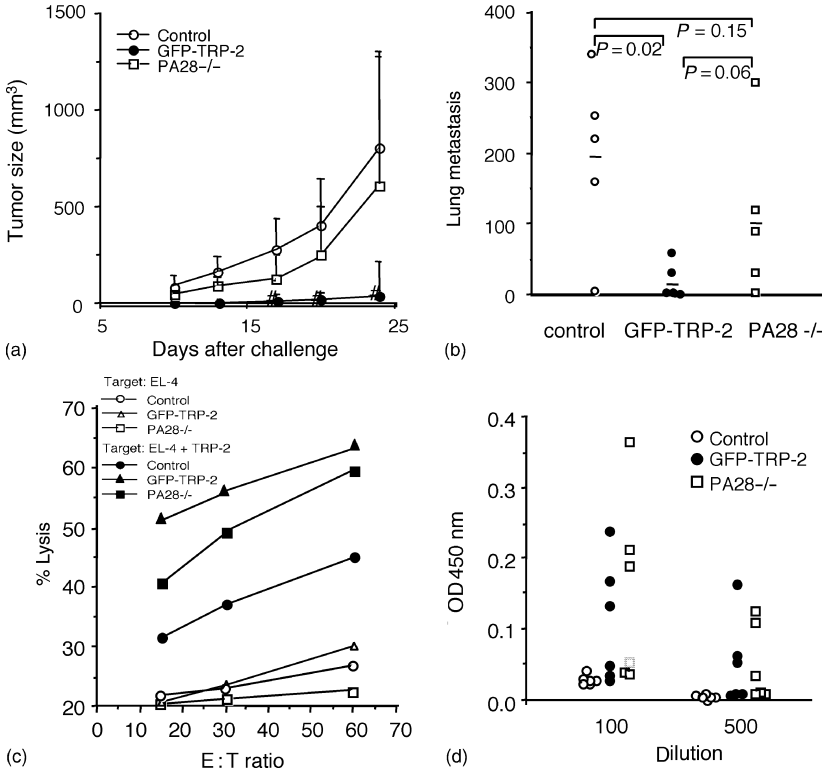

Figure 7. Defective induction of antitumour immunity by immunization with pGFP-TRP-2 in PA28$\alpha/\beta^{-/-}$ mice.C57BL/6 (closed circles) and PA28$\alpha/\beta^{-/-}$ knockout mice (open squares) immunized with pGFP-TRP-2 as well as unvaccinated control mice (open circle) were challenged s.c. with 2×10^5 B16F1 cells, and tumour growth was investigated (a). Number of lung metastases in mice immunized with pGFP-TRP-2 (b). CTL activity in pGFP-TRP-2-immunized B6 and PA28$\alpha/\beta^{-/-}$ mice (c). GFP-specific IgG was detected by ELISA in pGFP-TRP-2-immunized B6 and PA28$\alpha/\beta^{-/-}$ mice (d). Each value represents the mean ± SD from seven mice. #$P < 0{\cdot}01$ compared with the other two groups, by unpaired Student's *t*-test. This experiment was repeated three times with similar results.

pathway[1] should play an indispensable role in the present vaccination system. These results agree with the report by Steitz *et al.*[18] They showed that immunization with a plasmid encoding a fusion protein of GFP and mTRP-2_{30-519} could induce tumour-specific protective immunity promoted by TRP-$2_{180-188}$-specific $CD8^+$ T cells as evaluated by interferon-γ production through being activated by GFP-specific $CD4^+$ T cells, although they did not assay CTL activity. In their experiments using adenovirus vector encoding the fusion protein, effective antitumour immunity against lung metastasis of B16 melanoma was induced.

Many melanoma tumour antigens have been identified in the last decade because of the development of new technology. A solid base for antigen-specific cancer immunotherapy has been established by approaches such as immunization with antigenic peptides,[21] immunization with dendritic cells pulsed with tumour antigens,[22] and immunization with a naked DNA or recombinant viral vaccines.[23] Immunization with plasmid DNA has some advantages compared with other approaches: it is safe, readily deliverable, molecularly defined and can easily be constructed to enhance antigenicity by various means such as enhancement of antigen processing or adjuvant activity through a CpG motif included in plasmid DNA. We demonstrated here that vaccination of mice with a naked chimera DNA expressing a melanoma CTL epitope fused to GFP conferred protection against the tumour challenge and effectively induced epitope-specific CTLs. Immunization with DNA encoding peptide alone often failed to induce antitumour immunity effectively. The cause of this failure is still under investigation, although there exists at least four possible mechanisms. First, linked T-cell help may be required. Second, an N-terminal extension of a peptide epitope may simply prolong its biological half life. Third, cross-presentation of the construct by dendritic cells may require an antigen larger than the peptide epitope itself for either targeting to the processing pathway. Fourth, the N-terminus of the $CD8^+$ T-cell epitope may protect from degradation.

Taken together, our results indicate that fusion of a foreign protein to a CTL epitope plays dual roles in the induction of effective antitumour immunity. One role is activation of $CD4^+$ T cells that are required for induction of fully functional $CD8^+$ T cells, and the other role is facilitation of antigen presentation to $CD8^+$ T cells through the ubiquitin-proteasome pathway. This vaccine strategy using CTL epitopes fused with foreign proteins

would be applicable to human immunotherapy. It is required, however, to seek suitable foreign proteins that are readily processed by the UFD pathway.

ACKNOWLEDGMENTS

This work was supported by grants-in-aid from the Ministry of Education, Science, Sport, and Culture of Japan (15019075, 15025255, 15390136, 15659265).

REFERENCES

1 Tanaka K, Kasahara M. The MHC class I ligand-generating system: roles of immunoproteasomes and interferon-γ-inducible proteasome activator PA28. Immunol Rev 1998; **163:** 161–76.
2 Doherty FJ, Dawson S, Mayer RJ. The ubiquitin-proteasome pathway of intracellular proteolysis. Essays Biochem 2002; **38:**51–63.
3 Pickart CM. Mechanisms underlying ubiquitination. Annu Rev Biochem 2001; **70:**503–33.
4 Henderson RA, Finn OJ. Human tumor antigens are ready to fly. Adv Immunol 1996; **62:**217–56.
5 van den Eynde BJ, van der Bruggen P. T cell defined tumor antigens. Curr Opin Immunol 1997; **9:**684–93.
6 Rosenberg SA. A new era for cancer immunotherapy based on the genes that encode cancer antigens. Immunity 1999; **10:**281–7.
7 Wang RF, Appella E, Kawakami Y, Kang X, Rosenberg SA. Identification of TRP-2 as a human tumor antigen recognized by cytotoxic T lymphocytes. J Exp Med 1996; **184:**2207–16.
8 Bloom MB, Perry-Lalley D, Robbins PF, Li Y, el-Gamil M, Rosenberg SA, Yang JC. Identification of tyrosinase-related protein 2 as a tumor rejection antigen for the B16 melanoma. J Exp Med 1997; **185:**453–9.
9 Kawakami Y, Robbins PF, Wang RF, Parkhurst M, Kang X, Rosenberg SA. The use of melanosomal proteins in the immunotherapy of melanoma. J Immunother 1998; **21:**237–46.
10 Ashton-Rickardt PG, Bandeira A, Delaney JR, Van Kaer L, Pircher HP, Zinkernagel RM, Tonegawa S. Evidence for differential avidity model of T cell selection in the thymus. Cell 1994; **76:**651–63.
11 Inaba K, Young JW, Steinman RM. Direct activation of $CD8^+$ cytotoxic T lymphocytes by dendritic cells. J Exp Med 1987; **166:**182–94.
12 Sato M, Chamoto K, Tsuji T, Iwakura Y, Togashi Y, Koda T, Nishimura T. Th1 cytokine-conditioned bone marrow-derived dendritic cells can bypass the requirement for Th functions during the generation of $CD8^+$ CTL. J Immunol 2001; **167:**3687–91.
13 Tuting T, Gambotto A, DeLeo A, Lotze MT, Robbins PD, Storkus WJ. Induction of tumor antigen-specific immunity using plasmid DNA immunization in mice. Cancer Gene Ther 1999; **6:**73–80.
14 Dantuma NP, Lindsten K, Glas R, Jellne M, Masucci MG. Short-lived green fluorescent proteins for quantifying ubiquitin/proteasome-dependent proteolysis in living cells. Nature Biotechnol 2000; **18:**538–43.
15 Murata S, Udono H, Tanahashi N *et al*. Immunoproteasome assembly and antigen presentation in mice lacking both PA28α and PA28β. EMBO J 2001; **20:**5898–907.
16 Sakai T, Hisaeda H, Nakano Y *et al*. Gene gun-mediated delivery of an interleukin-12 expression plasmid protects against infections with the intracellular protozoan parasites *Leishmania* major and *Trypanosoma cruzi* in mice. Immunol 2000; **99:**615–24.
17 Sakai T, Hisaeda H, Nakano Y *et al*. Gene gun-based co-immunization of merozoit surface protein-1 cDNA with IL-12 expression plasmid confers protection against lethal *Plasmodium yoelii* in A/J mice. Vaccine 2003; **21:**1432–44.
18 Steitz J, Bruck J, Gambotto A, Knop J, Tuting T. Genetic immunization with a melanocytic self-antigen linked to foreign helper sequences breaks tolerance and induces autoimmunity and tumor immunity. Gene Ther 2002; **9:**208–13.
19 Hicke L. Protein regulation by monoubiquitin. Nat Rev Cell Biol 2001; **2:**195–201.
20 Cormack BP, Valdivia RH, Falkow S. FACS-optimized mutants of the green fluorescent protein (GFP). Gene 1996; **173:**33–8.
21 Marchand M, van Baren N, Weynants P *et al*. Tumor regressions observed in patients with metastatic melanoma treated with an antigenic peptide encoded by gene MAGE-3 and presented by HLA-A1. Int J Cancer 1999; **80:**219–30.
22 Nestle FO, Alijagic S, Gilliet M, Sun Y, Grabbe S, Dummer R, Burg G, Schadendorf D. Vaccination of melanoma patients with peptide- or tumor lysate-pulsed dendritic cells. Nat Med 1998; **4:**328–32.
23 Bronte V, Apolloni E, Ronca R, Zamboni P, Overwijk WW, Surman DR, Restifo NP, Zanovello P. Genetic vaccination with 'self' tyrosinase-related protein 2 causes melanoma eradication but not vitiligo. Cancer Res 2000; **60:**253–8.